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The aim of this project is to define the second messengers responsible for the regulation of microvascular endothelial cell structure and function and to determine the mechanisms by which ischemia and reperfusion alters these messengers to produce injury to the skin. This report reviews results obtained in the following areas: A. cyclic AMP, calcium, and protein kinase C in the regulation of neovascularization under normal and ischemic growth conditions; B. the mechanisms by which protein kinase C, calcium, and vimentin are coordinated to regulate microvascular endothelial cell shape and function; C. molecular mechanisms responsible for regulation of endothelial cell structure; D role of neutrophils and ischemia in producing endothelial cell damage; and E. dexamethasone and lipopolysaccharide in neutrophil damage to microvascular endothelial cells.

The techniques that we used in this project include phase contrast and electron microscopy, digital imaging fluorescence microscopy, immunocytochemistry to detect changes in the cytoskeletal proteins; and northern blot analysis for the determination of protooncogene activation.

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ROLE OF SECOND MESSENGERS IN ISCHEMIC TISSUE DAMAGE

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THE ROLE OF CYCLIC AMP, CALCIUM AND PROTEIN KINASE C, IN NEOVASCULARIZATION UNDER NORMAL AND ISCHEMIC GROWTH CONDITIONS

A. DESCRIPTION

Neovascularization plays a central role in wound healing. Ischemia is considered to be one of the factors that triggers this process. In this study we have examined how activators of protein kinase C affect neovascularization, and if ischemia alters the response to these activators.

Angiogenesis was induced in monolayer cultures by exposure of the apical surface of the culture to a collagen gel. Under these conditions, endothelial cells reorganize into vessel-like structures. Phorbol myristate acetate was used to stimulate neovascularization and 5-isoquinolinysulfonyl 2-methyl piperazine (H-7) and staurosporine was used to inhibit protein kinase C. Prior to stimulation with PMA, cells were pulsed with 32-Phosphate, and phosphorylated proteins were analyzed by PAGE. In parallel experiments, cells were kept under ischemic conditions for 18 hours prior to the induction of neovascularization with collagen.

B. RESULTS

1. Effect of exogenous cAMP on in vitro angiogenesis

Cyclic AMP markedly stimulates endothelial cell proliferation and viability in monolayer cultures. Cells with exogenous dibutyryl cAMP (5 X 10^{-4} M) in the medium grew at 1.5 times the rate of cells with cAMP (Fig 1). Cell migration and reorganization into vessels was significantly inhibted by dibutyryl cAMP. After 1 hour in the absence of cAMP, 60% of the cells remained in stage 1 (original monolayer) and 40% were entering stage 2 (migrating). In contrast approximately 80% of cells with cAMP were still in the original monolayer and only 20% of the cells entered stage 2, (Fig. 2, 3, 6A,

7A). cAMP also prolongs the integrity of the cells in vessel formation from 16-24 hours depending on the cell line (Fig 2,3). Exogenous cAMP slowed the formation of vessels; however, once the cells reorganized cell life was increased.

2. Effect of calcium on in vitro angiogenesis

To determine if the second messenger calcium altered vessel formation, angiogenesis was measured in the presence of both high and low calcium concentrations, and in the presence of the calcium ionophore A23187 and the chanel blocker verapamil. The increased calcium concentration did not alter the kinetics of vessel formation. Addition of A23187 caused cells to round and release from the dish.

3. Effect of phorbol myristate acetate (PMA), H-7 and staurosporine on angiogenesis.

Activation of protein kinase C with PMA increased the rate of migration and prolonged vessel lifetime in a dose-dependent manner. When treated with PMA the cells reorganized into vessels on average 10 hours faster than control and remained intact 48 hours longer (Fig. 4). Two PKC inhibitors were used to study the effect of blocking the action of PKC. Cells grown for 1 hour with H-7 (10^{-6} M) remained in a confluent monolayer and did not respond to collagen overlay (Fig. 4, 6C, 7C, 8C). Cells with both PMA and inhibitor showed shape changes but no migration (Fig 6D, 7D, 8D). The inhibition of angiogenesis was reversed immediately when the inhibitor was removed from the media.

4. Effects of PMA, H-7 and staurosporine on PKC activity

To verify that PMA, H-7, and staurosporine directly affected PKC activity, protein kinase c activity was measured directly using the transfer of gamma labeled P-32 to type III histone. The results

demonstrated that PMA activates PKC by 100 % while H-7 inhibits PMA activation by 45% (Fig. 5)

5. Effects of PMA and staurosporine on endogenous protein phosphorylation.

To determine what proteins are phosphorylated by PKC, we determined the incorporation of P-32 into proteins in the presence of PMA, staurosporine and PMA and staurosporine by slab gel electrophoresis and Western blot analysis of cytoskeletal proteins. On 2 dimensional gels 5 distinct bands were altered by treatment with PMA. The bands appeared at 82, 75, 60, 58, and 55 dKa (Fig. 9). As seen in Fig 9, a distinct change in phosphorylation occurred at the spot corresponding to that of vimentin (58 kD).

6. Effects of ischemia on new vessel formation.

DMEC were kept under ischemic conditions for periods ranging from 3 hours to 18 hours, and the rates of new vessel formation compared to controls. No differences in the kinetics of vessel formation were observed.

MECHANISMS BY WHICH CALCIUM, PROTEIN KINASE C AND VIMENTIN ARE COORDINATED IN MICROVASCULAR ENDOTHELIAL CELLS

A. DESCRIPTION

Major cytoskeletal changes of microvascular endothelial cells are observed after activation. To understand the mechanism of these changes, the functional-structural relationships of calcium, protein kinase C and vimentin in histamine-treated and control DMEC were studied.

The distribution of vimentin and protein kinase C was monitored with monoclonal antibodies. Changes in the location and kinetics of

calcium were studied using the calcium probe Fluo-3 in conjunction with interactive digital laser cytometry.

B. RESULTS

Vimentin in control DMEC is characteristically deployed as both a coarse reticular perinuclear matrix and a fine radial meshwork in the cell periphery. PKC is located in highest concentration within the nucleus, and secondarily in the perinuclear zone delimited by the reticular vimentin. Little free calcium is seen in the nucleus or cytoplasm. Histamine produces a striking 3X increase in nuclear calcium and a 2x increase in the perinuclear zone within 10 sec. The perinuclear reticular vimentin zone subsequently disappears, while the finer periperhal zone vimentin reorganizes as linear arrays along the long axis of the cells.

The spatial coordination of calcium, PKC and vimentin and their changes in response to histamine activation are unique to DMEC and are not observed in keratinocytes, melanocytes or fibroblasts. PKC location within the nucleus and perinuclear zone, the compartmentalized increase of calcium within these same zones and the disassembly of the perinuclear reticular vimentin matrix in response to histamine are all consistent with a major role for protein kinase C and its activation by calcium in initiating the morphological changes observed in the earliest stages of skin inflammation and neovascularization.

MOLECULAR MECHANISMS RESPONSIBLE FOR REGULATION OF ENDOTHELIAL CELL SHAPE

A. DESCRIPTION

Human dermal microvascular endothelial cells change shape dramatically when activated with the inflammatory agent phorbol myristate acetate both in vivo and in vitro. This change in shape constitutes one of the first changes in the response of the

endothelium to PMNs and to the sprouting observed during angiogenesis. In vitro, this shape change is closely regulated by the levels of cyclic AMP and calcium within the cell. Agonists which activate adenyl cyclase maintain cells in the epitheliold configuration, while those which increase intracellular calcium induce the mesenchymal transitions. These transitions in vitro are similar to those observed in the formation of granulation tissue in vivo and in the spindle shaped cells seen in Kaposi's sarcoma. Of major clinical importance is the role of the microvascular endothelial cell in tissue fibrosis. As a result of our studies on the factors that regulate endothelial cell shape, we have developed the hypothesis that the microvascular endothelial cell in all organs, when chronically damaged, may revert to a spindle-shaped cell responsible for tissue fibrosis. The way the protooncogenes are controlled in the microvascular endothelial cell may provide the answer in preventing these transitions which are a major cause of tissue destruction.

In these studies we have used probes for the oncogenes H-ras, N-ras, c-sis, c-myc and c-fos. Subconfluent 100 mm dishes of normal human dermal microvascular endothelial cells were treated with 2 uM PMA for various times so that all cultures were harvested within 30 minutes. The washed cultures were dissolved in 4 M guanidine thiocyanate, 0.1 M 2-mercaptoethanol, and centrifuged through a 5.7 M CsCl cushion at 36,000 rpm for 24 h at 20° C. The supernatant was carefully removed with a number of washes of 70% ethanol, and the pellet dried in vacuo. After solution in water the RNA was precipitated with 2.5 vol alcohol in 0.2 M potassium acetate pH 5.1. The RNA was collected and 15 ug portions electrophoresed on 1.2-1.5% agarose gels, washed with 60 mM NaOH, twice with 10 X SSC, and transferred to Optibind NC by capillarity using 10X SSC. The RNA was cross-linked to the membrane by means of a Stratalinker.

The membranes were probed at 67° C with each of the radioactive oncogene probes. In all experiments randomly primed DNA probes

were used. After washing at a stringency of 0.I SSC at 65° C, radioautographs were quantitated by densitometry and all values normalized to beta actin.

B. RESULTS

Transcription of c-fos was absent in microvascular endothelial cells but was rapidly induced following the addition of PMA, first appearing at 30 minutes and reaching a peak by I h. By 2 h it was no longer expressed and showed no subsequent induction within 32 h. In contrast, c-myc showed no stimulation and declined with an apparent half life of 4.4 h over the first 8 hr. Expression of c-cis was prominent in cultured endothelial cells, and declined 68% in I6 hours when treated with PMA. A similar result was obtained when cAMP was withdrawn from the growth medium. H-ras was present in cultured endothelial cells, but showed no change after PMA treatment. N-ras was not detected.

Protein kinase C is an element of the cellular signal transduction system which lies functionally in an intermediate position between cell surface receptors and gene expression. As we have described above, protein kinase C is present in the nucleus and perinuclear regions. These are the same regions which show calcium increases and shape changes when activated by histamine. Protein kinase C is known to phosphorylate vimentin to cause dissolution of the intermediate filaments of the cytoskeleton. As described above, we have demonstrated by means of immunocytochemistry striking changes in the distribution of vimentin following withdrawal of cyclic AMP. The inhibition of sis expression is interesting for it may be one of the controlling genes in angiogenesis. Activation of protein kinase C has been shown to be involved both in pathways which stimulate c-cis expression, such as IL-I beta and thrombin, and pathways which inhibit it, such as gamma interferon. The half life for the loss of sis mRNA is about I2 hours which compares with a value of 70-90 min reported in cAMP treated cells by other investigators. Unless the mRNA is

markedly stabilized under these condition, the decline in expression appears to result from a gradual and progressive repression of transcription, perhaps secondary to the alteration in differentiation reflected in the morphological changes.

ROLE OF NEUTROPHILS AND ISCHEMIA IN PRODUCING ENDOTHELIAL CELL DAMAGE

A. DESCRIPTION

Since there is increasing evidence that prior depletion of neutrophils by means of polyclonal antibodies, monoclonal antibodies, or by blocking of adhesion sites on the surface of neutrophils significantly reduces the damage to the microvasculartur e following ischemic reperfusion, we have investigated the interaction of neutrophils with endothelial cells under both normal and anoxic conditions, and following stimulation of cells with IL-I.

Neutrophils were isolated from normal blood by Ficoll-Hypaque gradient centrifugation. Endothelial cells were stimulated with I.0 units of IL-I per ml for 3 hours prior to the addition of neutrophils. Cells were kept under anoxic conditions for I8 hours prior to the stimulation with IL-I. Following a I hour interaction of neutrophils and endothelial cells, non-bound neutrophils were removed by gentle washing, the cultures fixed and stained with Giemsa, and the number of neutrophils bound to endothelial cells was determined by direct counting of 5 random fields by two investigators in a blind fashion.

B. RESULTS

Exposure of endothelial cells to anoxic conditions for time periods up to I8 hours did not significantly increase the binding of neutrophils to endothelial cells (Table 1). Endothelial cells remained viable under these conditions. Preincubation of cells with

10 units/ml of IL-1 for a period as short as 3 hours resulted in a striking increase in neutrophil binding (Table 2). When exposed to both anoxic conditions and IL-1, anoxia significantly increased the adhesion of neutrophils above that of either anoxia or IL-1 alone (Table 3). This was the first demonstration for how ischemic conditions may increase neutrophil adherence and damage via release of activated oxygen. Following binding of neutrophils, rapid lysis of the endothelial cell monolayer followed so that after 24 hours, no viable endothelial cells were observed.

OTHER FACTORS AFFECTING NEUTROPHIL BINDING: DEXAMETHASONE AND LIPOPOLYSACCHARIDE.

A. DESCRIPTION

The corticosteroids are highly effective in preventing inflammation and damage caused by neutrophil adherence to the vasculature. The mechanism by which corticosteroids prevent this damage is unknown. In this part of the study of endothelial cell damage, we have attempted to determine if the in vitro models we have developed could be used to answer this question, and we have examined the effects of dexamethasone on the capacity of cytokine treated DMEC to bind human PMNs and on LPS treated monocytes to release cytokines. At the same time we have also studied the effect of H-7 (a protein kinase inhibitor) and chlorpromazine (a calmodulin antgagonist) on the binding of neutrophils to DMEC.
Monoctye-enriched cell populations were prepared from the mononuclear cell fraction by density gradient centrifugation after stepwise increases in osmolarity of the cell suspension. To generate cytokin-enriched conditioned medium, one million monocytes were incubed with lipopolysaccharide (LPS 5 ug/ml), dexamethasone (20 ug/ml) or both at 37° C in a humidified incubator. After 4 hours all flasks were washed with RPMI and incubated for an additional 4 hours in complete Iscove's medium. Cytokine-enriched media were collected, centrifuged, and frozen at -80 C.

To induce adhesion molecules on DMEC, the cells were pretreated with cytokines released from monocytes, with IL-I (I0 u/ml). TNF-alpha (I000 u/ml). dexamethasone (20 ug/ml), H-7 (20 umole/ml), chlorpromazine(20 umole/ml) or in combination for 4 hours in quadruplicate wells. Isolated PMNs at a concentration of 50 PMNs to I DMEC were added. Monolayers were washed with PBS, and the number of bound PMNs counted.

B. RESULTS

The results of these studied demonstrated that conditioned medium from LPS-stimulated monocytes increased by 3 fold the number of PMNs bound to endothelial cells compared to controls. Dexamethasone and LPS co-treated monocyte conditioned medium showed a 30% decrease in DMEC adhesivity for PMNs compared with LPS-stimulated monocyte conditioned medium. Thus dexamethasone is, in some way, able to inhibit the generation of a cytokine by stimulated monocytes, and can provide us for the first time with a model with which to further study this important aspect of inflammation and its regulation. That protein kinase C is in some way involved in the interaction of neutrophils with DMEC was demonstrated by the finding that H-7 was able to significantly inhibit the binding of neutrophils in cytokine-enriched conditioned medium. Neither dexamethasone nor chlorpromazine showed similar effects.

A highly significant finding was the striking change in morphology that occurred when cytokine-enriched medium was added to DMEC monolayers. The cells immediately converted from the classical epitheliod morphology to the spindle-shaped morpholoy seen in the earliest stages of angiogenesis.

The ability of H-7 to block neutrophil adhesion suggests that this agent may be of use in helping to prevent the damage produced by neutrophils following ischemic reperfusion.

SUMMARY AND CONCLUSIONS

During the past year we have attempted to understand the factors that produce damage to the microvascular endothelial cell, edema, and skin death. It is now clear that in vitro, ischemia alone is not sufficient to account for the striking changes observed in the vasculature following brief periods of ischemia and reperfusion.

Edema is one of the consequences of ischemic injury, and the continuity of the endothelial cell lining is essential in preventing fluid leakage. The continuity of the endothelium is delicately balanced by two second messengers: cyclic AMP and calcium. Agonists which increase or maintain these levels stabilize the cytoskeleton, and the cells remain in the epitheliod configuration required for normal homeostasis. Agonists that increase calcium levels cause a contraction of the microvasculture leading to fluid leakage. Phosphoylation of vimentin by protein kinase C present in the nucleus and perinuclear regions of the endothelial cell appears to be the mechanism responsible for these changes. Inhibition of protein kinase C by H-7 completely inhibits the phenotypic changes induced by the activation of protein kinase C by phorbol esters. In addition to activation of protein kinase C, TPA markedly stimulates c-fos and inhibits c-myc expression. How these changes are integrated will provide an explanation for how the microvasculature insures integrity, and how cytokines produce the physiologic changes seen in neovascularization and inflammation.

Ischemia alone is also not able to increase the binding of neutrophils to endothelial monolayers. However, in the presence of IL-I, anoxia acts synergistically with IL-I to stimulate neutrophil binding, disruption of the cell monolayers, and destruction of the cells.

TABLE I

Aerobic vs. anaerobic binding of neutrophils to cultured microvascular endothelial cells

MEC condition	Mean number of MECs with 3 or more bound neutrophils
Aerobic	20
Anaerobic	27

TABLE II

The effect of IL-1 on MEC-neutrophil binding

MEC condition	Mean number of MECs with 3 or more bound neutrophils
Non-stimulated	30
IL-1 stimulated	281

TABLE III

The effect of 3-hour ischemia followed by reperfusion on MEC-neutrophil binding

Length of reperfusion	Mean number of normal MECs with 3 or more bound neutrophils	Mean number of IL-1 stimulated MECs with 3 or more bound neutrophils
0 h	20	319
.5 h	20	242
24 h	94	408
48 h	36	275
72 h	-	30

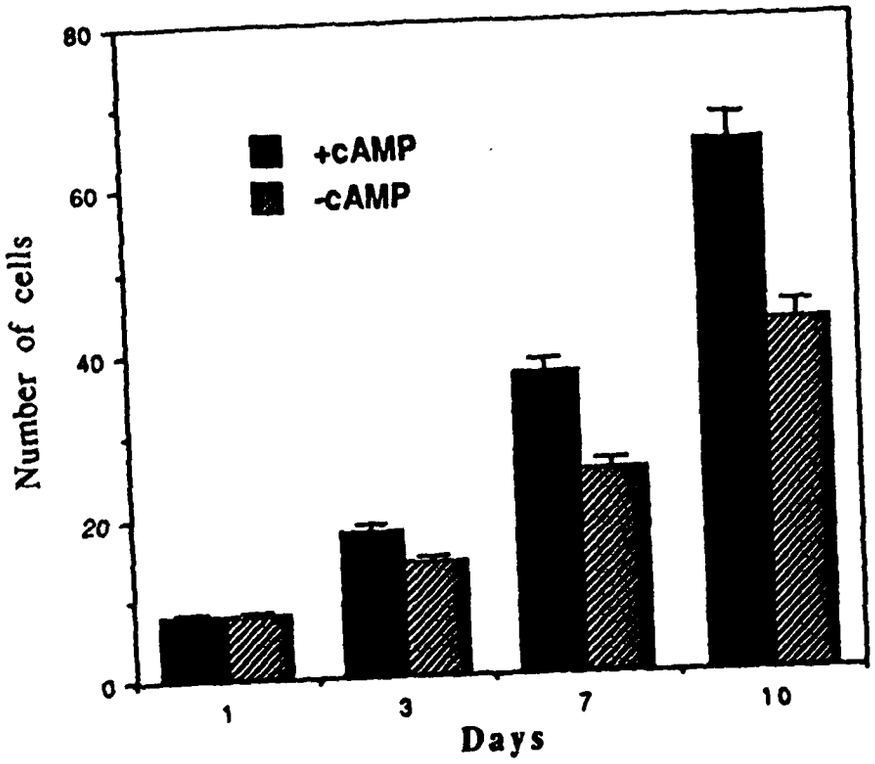

Fig. 1 Effect of dibutyryl cAMP on growth of endotheial cells in culture. DMEC were plated on 25 cm^2 corning dishes at 10 cells/cm^2. On indicated days cells were removed from dishes using 0.1% trypsin and counted using a hemocytometer.

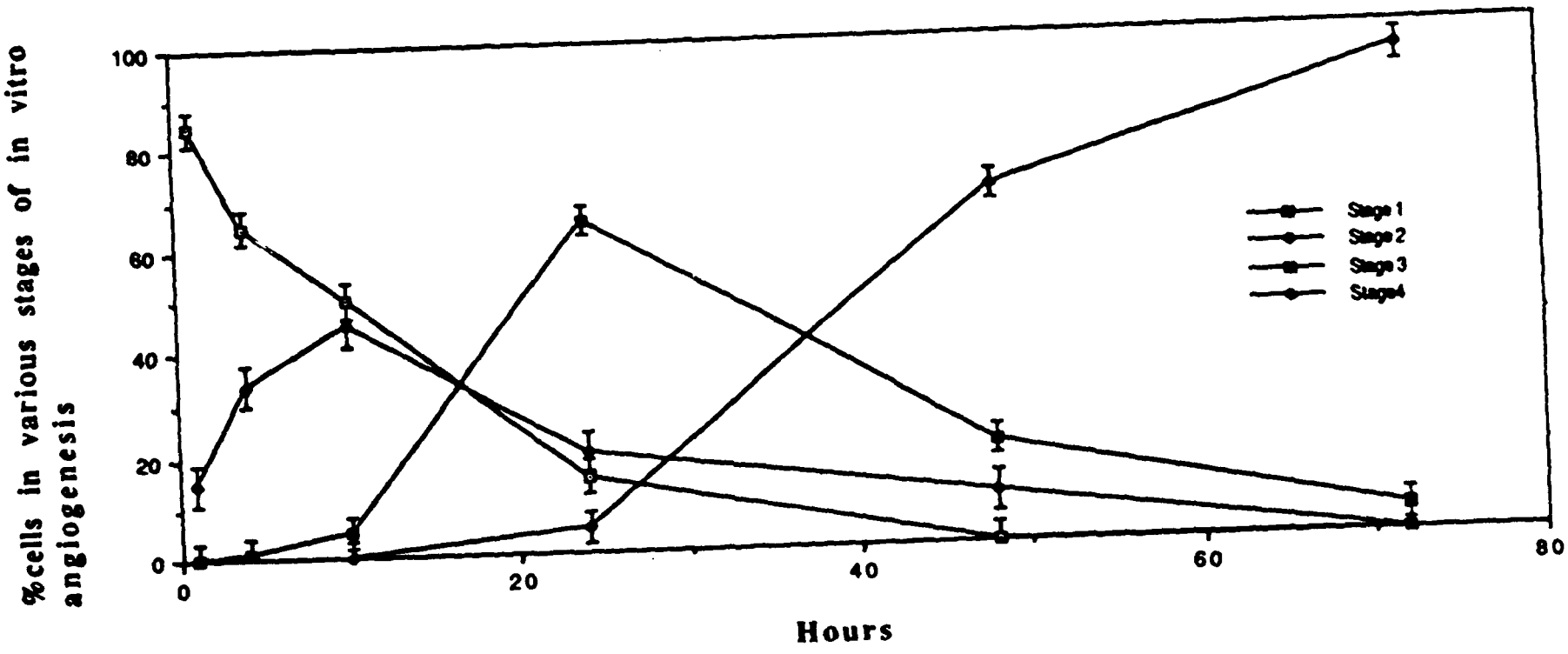

Fig. 2 The kinetics of in vitro vessel formation and degradation in the presence of dibutyryl cAMP

For Fig. 2,3,4 Definitions for the various stages of in vitro angiogenesis.
Stage 1-epithelioid monolayer.
Stage 2-conversion to spindle shaped migratory cells.
Stage 3- reallignment of spindle shaped cells into vascular channels with lumens.
Stage 4-cell lysis

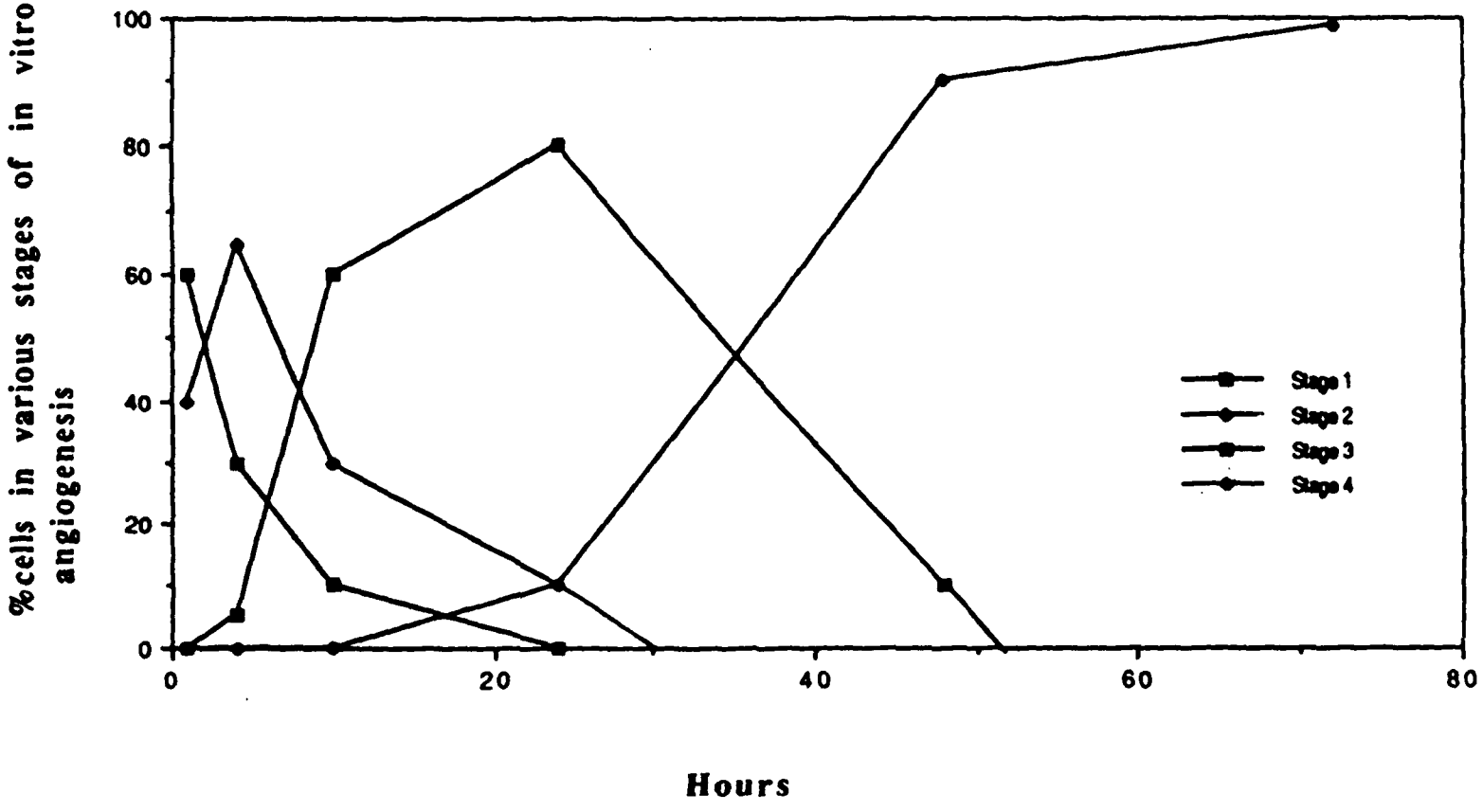

Fig. 3 The kinetics of in vitro vessel formation and degradation in the absence of dibutyryl cAMP.

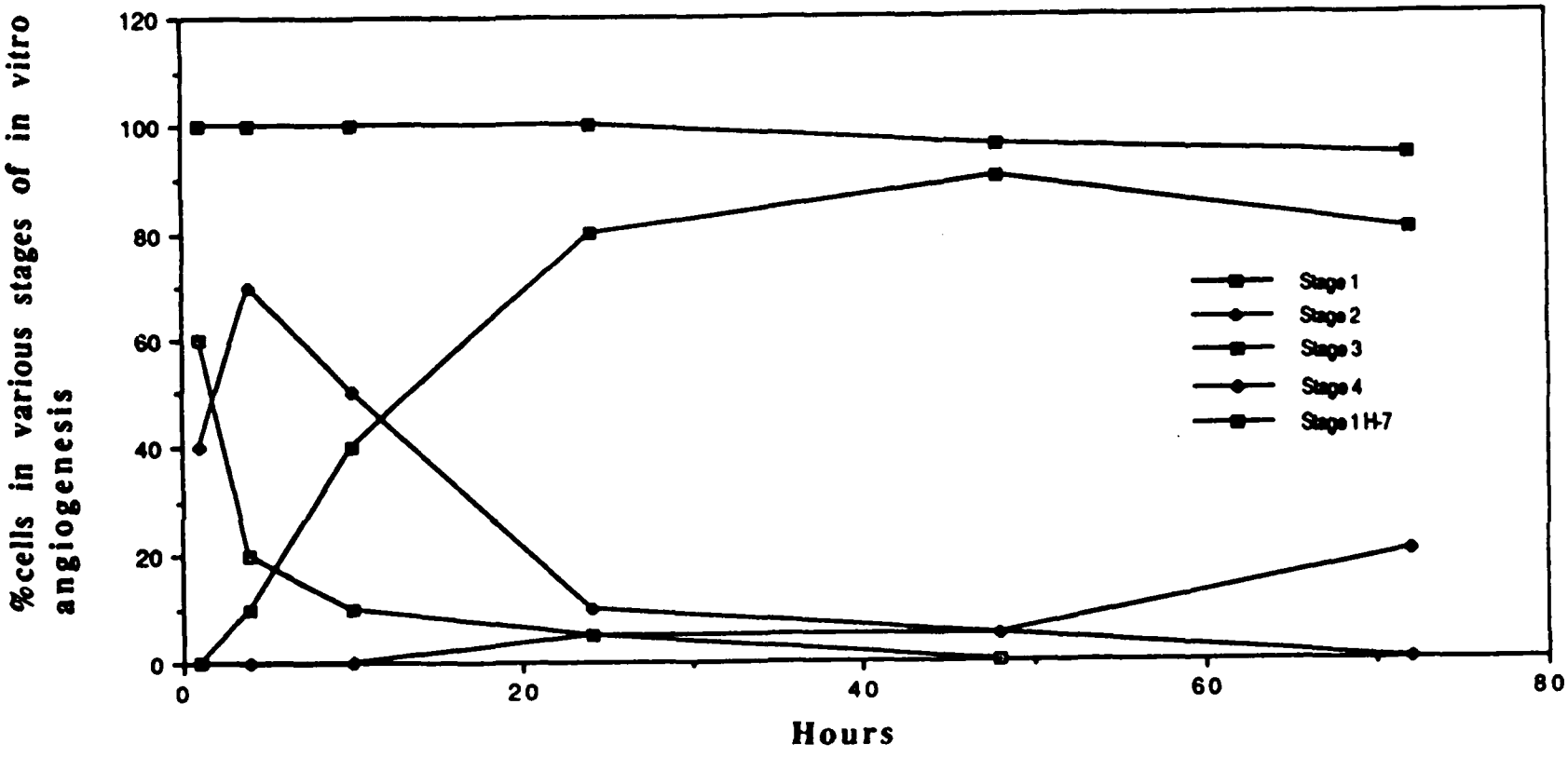

Fig. 4 Effect of H-7 and PMA on the kinetics of in vitro vessel formation

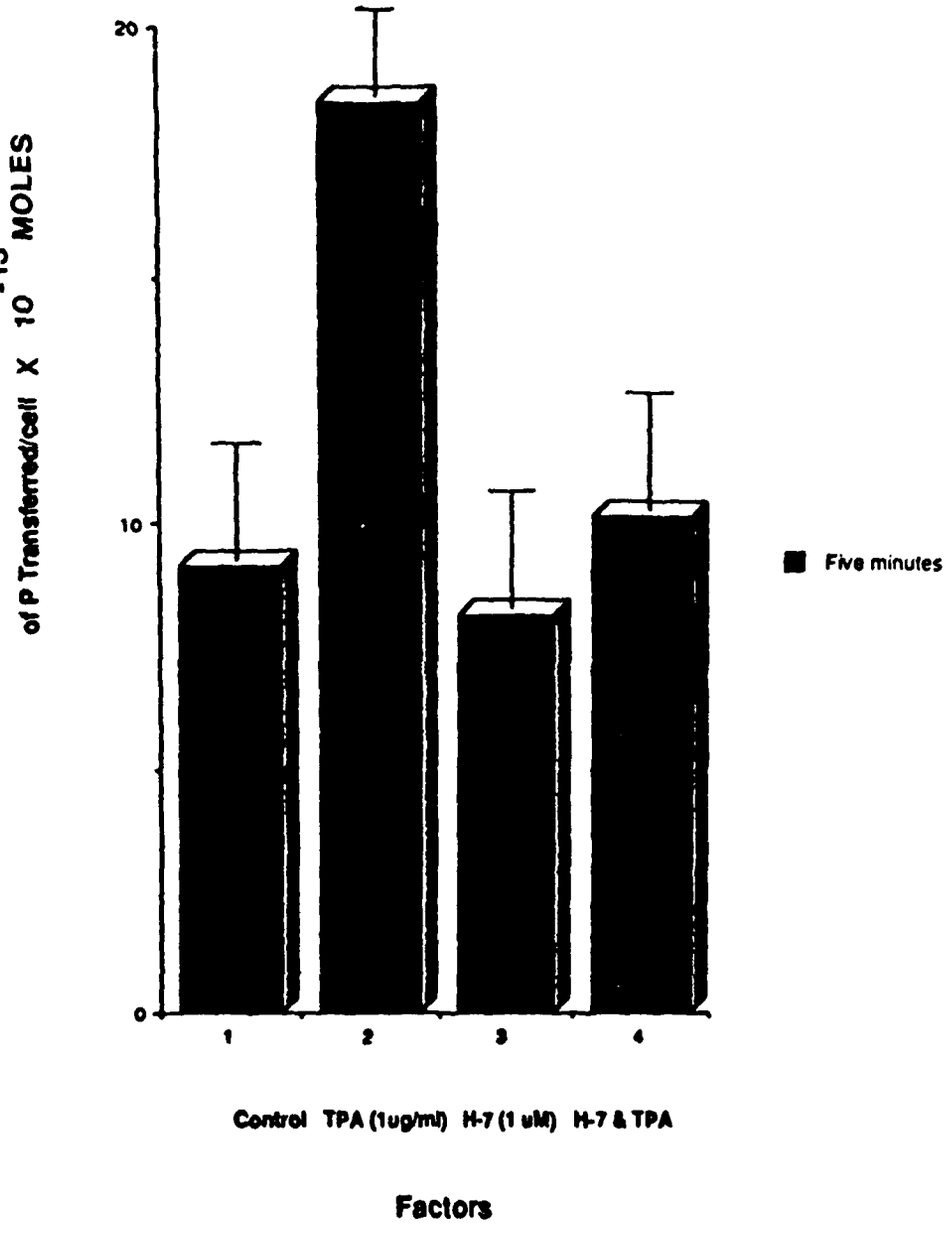

Fig. 5 Effect of PMA and H-7 on protein kinase C activity in DMEC.

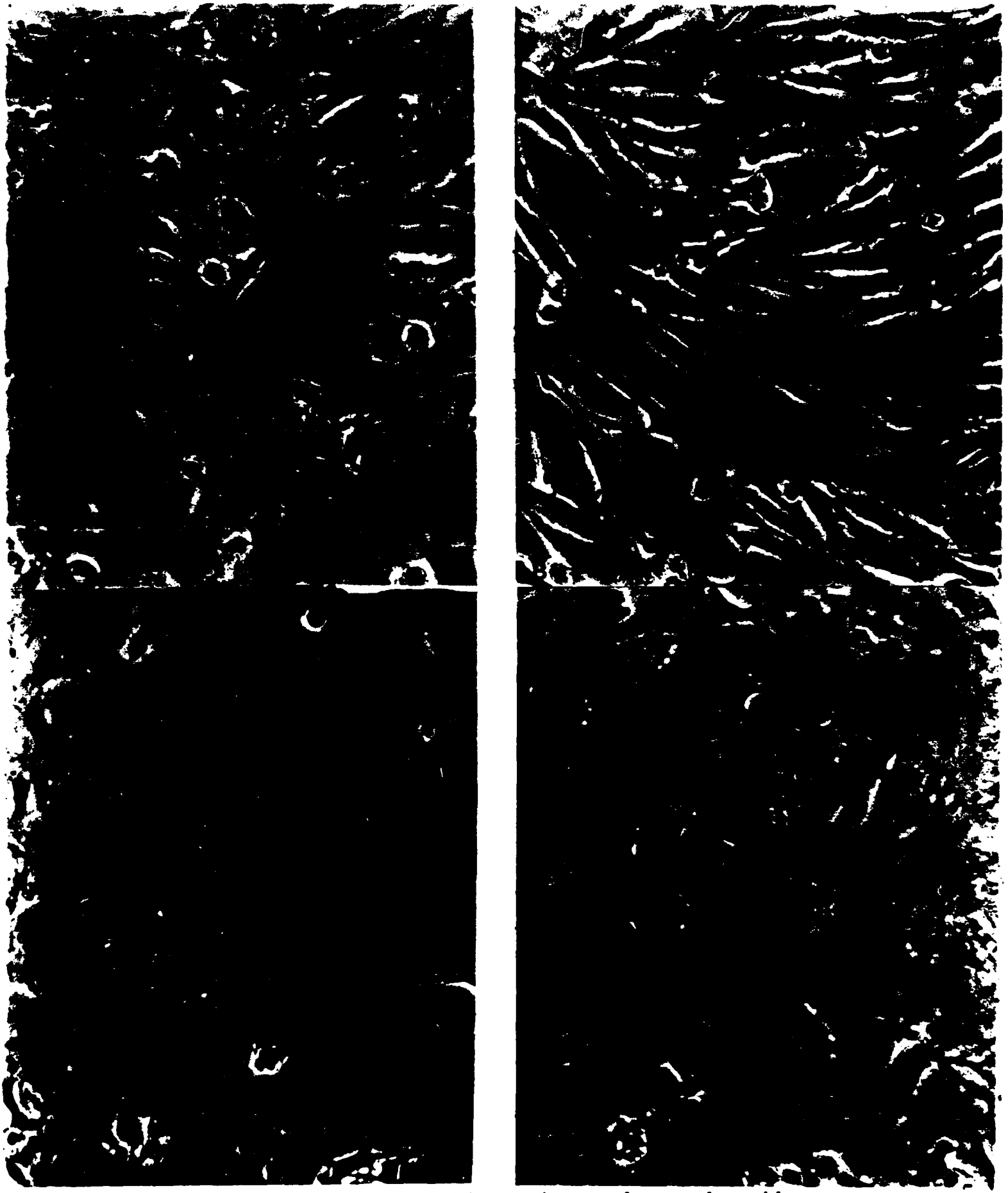

Fig. 6 Morphology of DMEC fifteen minutes after overlay with collagen in Iscove's complete media minus cAMP. H-7 and PMA were applied 4 hours prior to overlay. A. Control B. PMA(1 ug/ml) C. H-7(10-6 M) D. Both PMA and H-7.

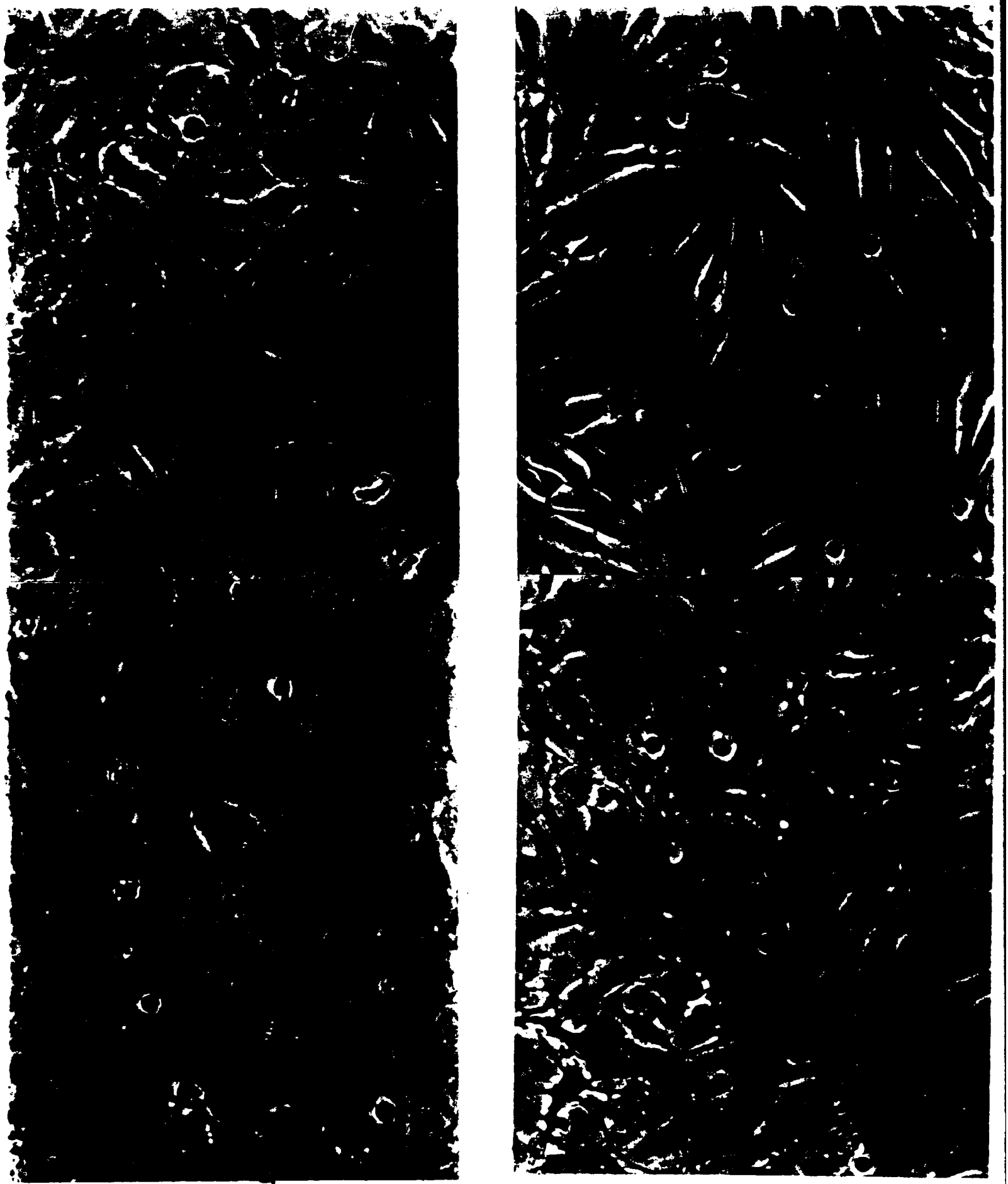

Fig. 7 Morphology of DMEC fifteen minutes after overlay with collagen in Iscove's complete plus cAMP. H-7 and PMA were added 4 hours prior to overlay. A. Control B. PMA(1 ug/ml) C. H-7(10-6 M) D. Both PMA and H-7.

Fig. 8 Morphology of DMEC 48 hours after overlay with collagen in Iscove's complete plus cAMP. H-7 and PMA were added 4 hours prior to overlay. A. Control B. PMA(1 ug/ml) C. H-7(10-6 M) D. Both PMA and H-7.

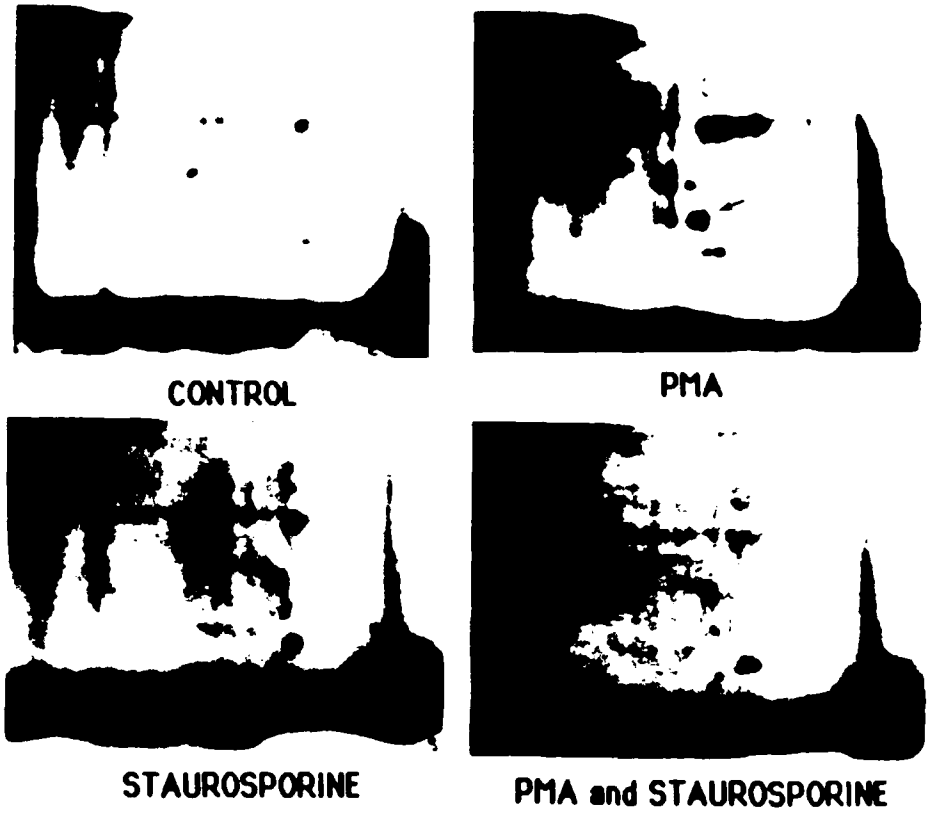

Fig. 9 Autoradiographs of 2-D SDS PAGE analysis of the effect of PKC activators and inhibitors on protein phosphorylation. Arrow signifies site corresponding to vimentin molecular weight.